High-Performance Liquid Chromotography Analysis of 8-Methoxypsoralen Monoadducts and Crosslinks in Lymphocytes and Keratinocytes

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Human lymphocytes and murine keratinocytes were treated with clinically relevant doses of 8-methoxypsoralen and long-wavelength ultraviolet radiation. The extent of 8-MOP photoadduct formation was determined by liquid scintillation analysis. The distribution of photoadducts was determined by HPLC analysis of enzymatically hydrolyzed DNA from these cells. Analysis of photoadduct formation in lymphocytes and keratinocytes showed that 4′,5′-monoadducts were the predominant photoadducts (42% and 41%, respectively). Monoadduct and crosslink yields were dependent on cell type as well as irradiating wavelength. The photochemical conversion of 400 nm-induced 4′,5′-monoadducts to crosslinks, as well as 3,4-monoadducts in poly(dA-dT), was measured. *J Invest Dermatol 97:151–155, 1991*

Eight-Methoxypsoralen (8-MOP) and long-wavelength ultraviolet radiation (UVA) combination photochemotherapy has been widely used for nearly two decades in the treatment of psoriasis [1]. More recently, 8-MOP and UVA have been used in an extracorporeal form to treat cutaneous T-cell lymphoma [2]. The proposed mechanism of action has implicated the formation of psoralen monoadducts and crosslinks, which have been presumed to be responsible for the modulation of the hyperproliferation of lymphocytes and keratinocytes [3]. However, the actual number and type of photoadducts formed in the target cells have never been reported. We have prepared monoclonal antibodies that recognize two of the 8-MOP photoadducts [4]. However, due to photoadduct cross-reactions and insufficient sensitivities, these antibodies have not been able to provide quantitative information regarding the extent and distribution of photoadducts formed in lymphocytes. Therefore, we have adapted an HPLC method used previously to detect and quantify photoadducts in synthetic polynucleotides [5] for the analysis of DNA from lymphocytes and keratinocytes treated with 8-MOP and UVA in vitro. The photoadducts formed in poly(dA-dT) were used to establish benchmark retention times. This information was then used to characterize photoadduct formation in enzymatically hydrolyzed DNA from cells that had been treated with 8-MOP and UVA. Photopheresis, in which lymphocytes are treated extracoporeally with 8-MOP and UVA, is analogous to the treatment of the same cells treated in petri dishes. Thus, the findings reported below should be directly applicable to that clinical situation.

The effects of several parameters were investigated. For example, longer wavelength UVA (400 nm) is usually employed to induce 4′,5′-monoadduct formation [6]. Subsequent irradiation at 350 nm is then used to induce crosslink formation. However, the extent of monoadduct conversion to crosslink has not been reported. In addition, the effects of 8-MOP and UVA on photoadduct yield in different cell types has not been studied. Because the actual numbers and types of photoadducts formed in cells have not been measured prior to these studies, it has not been possible to assign unequivocal roles to each photoadduct. The application of the techniques described in this paper to the various cell lines routinely exposed to 8-MOP and UVA may lead to interesting insights about the role of each photoadduct.

MATERIALS AND METHODS

Chemicals Methanol and water were HPLC-grade (Baker, Phillipsburg, NJ). All other chemicals were the highest purity available and were used without further purification.

Photoadduct Formation in Polynucleotides Solutions of poly(dA-dT) (Pharmacia, Piscataway, NJ) (1 mg/ml), 8-MOP (0.1 mg/ml), and [^{3}H]-8-MOP (5 μl/100 μl) (79 Ci/mmol, Amersham, Arlington Heights, IL) were prepared in 10 mM Tris, 1 mM EDTA, and 0.1 M NaCl. Solutions were irradiated at 400 nm (bandwidth, 10 nm) for 2 h at 25°C using a monochromator (Photon Technology Inc., Princeton, NJ). To remove unreacted 8-MOP and any degradation products, the polynucleotide solutions were made 0.4 M in LiCl and 2 volumes of cold ethanol were added to precipitate the DNA. Solutions were incubated at 0°C overnight, centrifuged at 10,000 rpm at 0°C for 30 min, and the supernatant removed. The DNA pellet was redissolved in distilled water. Half of the recovered polynucleotide was hydrolyzed immediately (see below) and the other half was irradiated at 350 nm (bandwidth, 10 nm) for 5 min to convert the 4′,5′-monoadducts to crosslinks, and the preparation was then hydrolyzed as described below.

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Abbreviations:
8-MOP: 8-methoxypsoralen
poly(dA-dT): self-complementary double stranded polynucleotide consisting of a copolymer of alternating adenosine and thymine nucleotides
UVA: long-wavelength ultraviolet radiation (320–400 nm)

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Isolation of Cells In these studies freshly isolated lymphocytes and keratinocytes, typically the targets of 8-MOP/UVA, were used.

Lymphocytes Whole blood was collected from normal human volunteers using LeucoPrep tubes containing EDTA (Becton-Dickinson, Lincoln Park, NJ). The blood was centrifuged at 3000 rpm for 15 min. The buffy coat containing the lymphocytes was removed and washed 3 times with PBS.

Murine Keratinocytes Ears from 6–8-month-old, anesthesized Balb c mice were split along the plane of the cartilage and then removed together with the subcutaneous tissue. The specimens were incubated for 1 h at 37°C in 0.25% trypsin. The epidermis was then separated from the dermis and the epidermal cells were dispersed in PBS containing 10% fetal calf serum by rubbing the separated epidermal sheets. After filtration through a cotton column, the cells were washed twice with PBS and the viability assessed by trypan blue exclusion (typically >80% for all samples).

Irradiation of Cells Lymphocytes or keratinocytes (3–5 million cells/ml) were suspended in PBS and treated with 8-MOP (1–200 ng/ml). Trace amounts of [^{3}H]8-MOP (Amersham) were added prior to irradiation with UVA in order to measure 8-MOP incorporation by liquid scintillometry. The cells were incubated in the presence of 8-MOP for 30 min in darkness at 4°C. To minimize repair during irradiation the cells were kept at 4°C during the irradiation period.

Cell suspensions were placed in plastic petri dishes and irradiated through UVB-absorbing window glass in a light box equipped with broad band UVA lamps (Westinghouse; peak emission at 355 nm). Total UVA doses (1–3 J/cm^2) were determined by radiometry (International Light, Newburyport, MA). These parameters were chosen to simulate the conditions to which lymphocytes and keratinocytes are typically exposed during photopheresis and PUVA, respectively. To eliminate the possibility of photoadduct repair during the irradiation step, the cells were treated at ~0°C, and all subsequent operations were performed at 4°C. Unirradiated cells served as dark controls.

After irradiation, cells were washed three times with PBS to remove unbound 8-MOP. DNA was extracted from keratinocytes immediately. For lymphocyte repair studies, DNA was extracted from one-half of the cells, and the remainder of the cells were placed in media (1640 RPMI with 10% fetal calf serum and 2% penicillin) at a final concentration of 3 million cells/ml and incubated at 37°C in a 5% CO_2 atmosphere. Cells were kept in culture until harvested at 48 h for DNA extraction.

DNA Extraction

Lymphocytes: Lymphocytes (typically 30–60 million) were suspended in 500 μl of 50 mM TRIS-150 mM NaCl-100 mM EDTA (pH 8). Nuclei were lysed with 40 μl of 10% SDS. Fifth microliters RNase A (10 mg/ml; Sigma) were added and the suspension was incubated for 30 min at 37°C. Two-hundred-fifty microliters 10% SDS were added and the solution was heat shocked at 60°C for 10 min. Fifty microliters 5 M sodium perchlorate were added, followed by 800 μl chloroform:isoamyl alcohol (24:1). The solution was inverted every 5 min for 30 min followed by centrifugation at 10,000 × *g* for 10 min. The aqueous phase was transferred to another tube, and 2–3 volumes of ice-cold 100% ethanol were added. The solution was centrifuged at 10,000 × *g* at 0°C for 30 min. The DNA pellet was washed with 500 μl water and kept on ice for 30 min to remove any soluble contaminants. The solution was made 0.3 M in sodium acetate, and 2–3 volumes of ice-cold 100% ethanol were added. After centrifugation at 10,000 × *g* at 0°C for 30 min, the supernatant was removed and the residual alcohol was allowed to evaporate. The DNA was resuspended in HPLC-grade water, and the concentration was determined by UV spectroscopy.

Keratinocytes: Keratinocytes (typically 40 million) were suspended in 2 ml 0.2 M NaCl–0.1 M TRIS-10 mM EDTA buffer (pH 8) and lysed by the addition of 25 μl 10% SDS. After a 30-min incubation at 37°C with RNase (100 μg/ml) and a 2-h incubation with proteinase K (200 μg/ml; Sigma), the lysates were made 1 M in NaCl. An equal volume of phenol/chloroform (1:1) was added; the samples were centrifuged at 1200 rpm for 20 min. The aqueous phase was then treated with chloroform/isoamyl alcohol, and the DNA was precipitated by the addition of NaCl followed by 2–3 volumes of cold ethanol. The DNA was resuspended in water and the concentration determined by UV spectroscopy.

DNA Characterization: The UV spectra of the DNA solutions were recorded, and DNA molar concentration in terms of nucleotide units or bases was computed from the absorbances at 260 and 280 nm. To assess the overall extent of 8-MOP incorporation, aliquots of the DNA samples (typically 100 μl) were assayed by liquid scintillation analysis. The specific activity of the 8-MOP was used to calculate the molar concentration of 8-MOP photoadducts in the DNA. The extent of photoadduct formation is reported as the number of photoadducts per million bases, which is obtained by dividing the molar 8-MOP concentration by the molar nucleotide concentration of the DNA sample.

Enzymatic Hydrolysis of DNA: Samples containing 50–250 μg of DNA were hydrolyzed. Solutions were made 15 mM in sodium acetate (pH 5). Twenty-five to fifty microliters of DNAase II (2 mg/ml) (Calbiochem, La Jolla, CA) were added, and samples were incubated for 24 h at 37°C, at which time a second aliquot of the enzyme was added for another 24 h. One molar Tris (pH 8) was used to adjust the pH to 7, and 20 μl phosphodiesterase II (20 units/ml) (Worthington, Freehold, NJ) were added. The mixture was incubated for 24 h at 37°C, at which time another aliquot of this enzyme was added and incubated for another 24 h. The pH was adjusted to 8 with 1 M Tris, and 10 μl of alkaline phosphatase (10,000 units/ml) (Worthington) were added. The mixture was then incubated at 37°C for 24 h, at which time a second aliquot was added for another 24 h. Prior to HPLC analysis, the samples were centrifuged [5].

HPLC Analysis: Aliquots of hydrolyzed DNA (150–300 μl) were applied to an octyldecylsilane (ODS) reversed-phase column (Regis, Morton, IL). Using a mobile phase consisting of water and methanol (see Fig 1 legend for details), the eluting species were monitored in two ways. First, the scanning wavelength detector was set at 260, 300, or 330 nm for the simultaneous detection of unmodified DNA nucleosides, as well as the psoralen-modified bases. Second, fractions collected during HPLC were analyzed by liquid scintillation spectrometry in order to detect species containing [^{3}H]8-MOP moieties. These data were used to compute the extent of photoadduct formation.

RESULTS

Poly(dA-dT) was irradiated at 400 nm for 2 h in the presence of 8-MOP and a trace amount of [^{3}H]-8MOP. Figure 1 shows the HPLC chromatogram of the enzymatically hydrolyzed DNA. In *A* the absorbance at 300 nm is plotted versus retention time and in *B* the percentage of counts per minute (CPM) derived from [^{3}H]8-MOP is plotted versus fraction number. The more intense scintillometric signals corresponding to 8-MOP–containing species were used to compute the photoadduct yields. The major photoproduct, a 4′,5′-monoadduct (66% yield), elutes at 27 min. The UV absorbance spectrum of this material was determined as the material passed through the flow cell of the scanning detector (Fig 2). The absorption maximum at 330 nm and the shoulder at 233 nm are consistent with published spectra of the 4′,5′-monoadduct [8]. The 3,4-monoadduct (21.48 min) and crosslink (17.82 min) formed to a lesser extent (14.5% and 19.5%, respectively) in 400 nm-irradiated poly(dA-dT). Their respective absorbance spectra are also shown in Fig 2.

Following the precipitation of the poly(dA-dT) and removal of unreacted 8-MOP, an aliquot of the monoadducted polynucleotide was re-irradiated at 350 nm. As shown in Fig 3*A*, the 4′,5′-monoadduct at 27 min is virtually eliminated and prominent peaks are evident at 17.6 and 19.7 min, which correspond to the newly

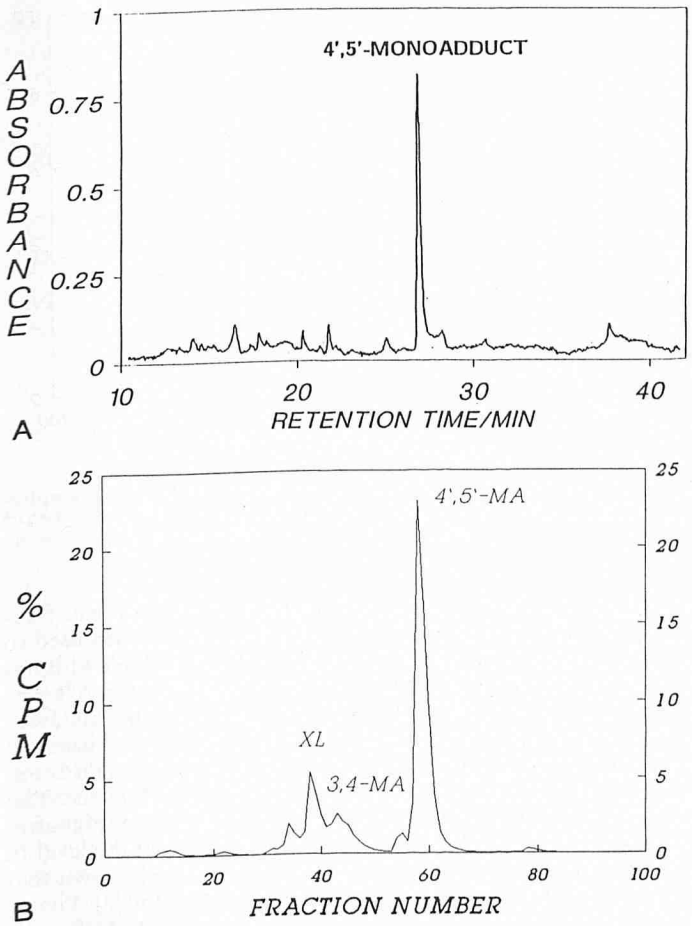

Figure 1. HPLC analysis of poly(dA-dT) after photomodification with 8-MOP and 400 nm radiation and enzymatic hydrolysis. *Upper panel,* UV absorbance (1 aufs) at 300 nm is plotted versus retention time. The gradient consisted of water and methanol: 0–5 min, 0% acetonitrile–100% water; 5–15 min, 0–30% acetonitrile; 15–20 min, 30% acetonitrile; 20–35 min, 30–70% acetonitrile; 35–40 min, 70% acetonitrile; 40–50 min, 70–100% acetonitrile (the same gradient was used in all subsequent analyses). *Lower panel,* Percent of total CPM versus fraction number (note: fractions were collected at 0.5 min intervals).

formed crosslink and 3,4-monoadduct, respectively. These data are plotted in Fig 3*B* as the percentage of total counts per minute (CPM) versus the HPLC fraction number. The peak corresponding to the 4′,5′-monoadduct was reduced by 93%, whereas the peaks corresponding to the crosslink and 3,4-monoadduct were increased by 212% and 147%, respectively (Table I).

The retention time of specific photoadducts formed in poly(dA-dT) was used to characterize the photoadduct formation in lymphocytes and keratinocytes. Human lymphocytes were treated in vitro with 100 ng/ml 8-MOP containing trace amounts of [^{3}H]-8-MOP, and 1 J/cm^2 UVA. The DNA was isolated, enzymatically hydrolyzed, and analyzed by HPLC and liquid scintillometry. Due to the low levels of 8-MOP photomodification that occurred in the treated cells and the small quantity of DNA that was available for analysis, the optical signals from the 8-MOP photoadducts were too weak to be detected. Figure 4 shows the percentage of total CPM versus the HPLC fraction number in DNA extracted from lymphocytes. The pattern of peaks is similar to that shown in Figs 1*B* and 3*B* for poly(dA-dT). Under these conditions, 19.9% of the adducts were 3,4-monoadducts, 42.2% were 4′,5′-monoadducts, and 37.9% were crosslinks (see Table II).

DNA from murine keratinocytes treated in vitro with 50 ng/ml of 8-MOP and 3 J/cm^2 UVA were also analyzed (Fig 5). The distribution of specific adducts differed slightly from the distribution seen in lymphocytes treated with 100 ng/ml 8-MOP and 1 J/cm^2 UVA. In the keratinocytes a higher percentage of the total adducts were 3,4-monoadducts (33.9% vs. 19.9%), whereas a lower percentage of the total adducts were crosslinks (24.9% vs. 37.9%) (see Table II). The percentage of 4′,5′-monoadducts in the two cell types was nearly identical. It should be noted that two extraneous peaks appear in the CPM figures. A peak near fraction 12 amounting to as much as 2–5% of the total counts is due to undigested DNA. Another peak near fraction 80 is due to trace amounts of free 8-MOP.

Recent work in our laboratory determined the 8-MOP and UVA

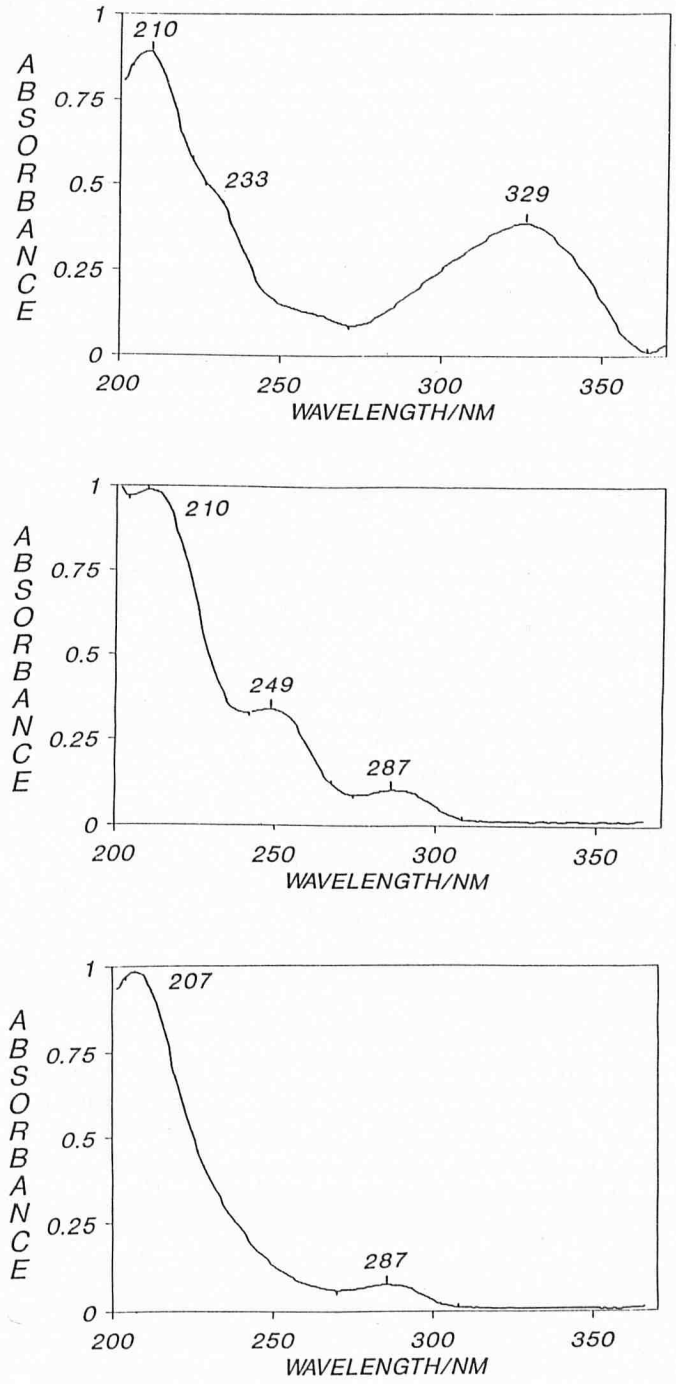

Figure 2. UV spectra of 8-MOP photoadducts obtained from the scanning detector during HPLC analysis of the hydrolyzed poly(dA-dT). *Upper panel,* 4′,5′-monoadduct; *middle panel,* 3,4-monoadduct; *lower panel,* crosslink.

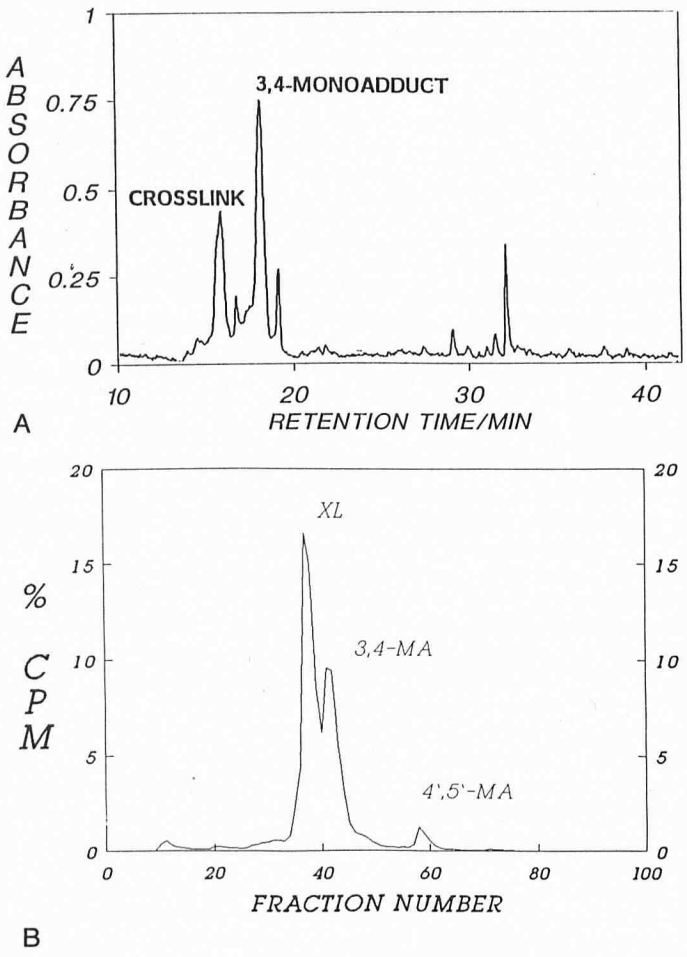

Figure 3. HPLC analysis of 400 nm-irradiated poly(dA-dT) after exposure to 350 nm radiation. *Upper panel,* UV absorbance (1 aufs) at 260 nm versus retention time; *lower panel,* percent of total CPM versus fraction number.

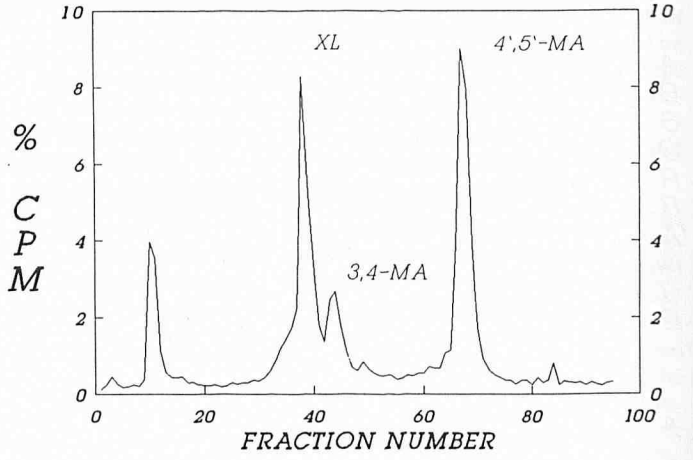

Figure 4. HPLC analysis of DNA from 8-MOP/UVA-treated lymphocytes. Percent of total CPM versus fraction number.

doses under which DNA repair occurred in lymphocytes [9]. Lymphocytes treated with 8-MOP and UVA and then placed in culture for 48 h showed a 25% reduction in the total number of photoadducts. When lymphocytes were treated with 10 ng/ml of 8-MOP and 1 J/cm² UVA, the overall number of adducts was reduced proportionately and in preliminary studies we have found that the distribution of adducts also changes (37%, 37%, and 26% for the 3,4-monoadduct, the 4′,5′-monoadduct and the crosslink, respectively).

DISCUSSION

Specific 8-MOP photoadducts in the DNA of human lymphocytes and murine keratinocytes treated in vitro with 8-MOP and UVA have been detected and quantified using reversed-phase HPLC. Photoadducts, formed with high efficiency in the synthetic polynucleotide, poly(dA-dT), were used to establish retention time standards for the 8-MOP photoadducts and the corresponding pattern of peaks in the plot of [³H]8-MOP scintillation counts versus fraction number. These characteristic peak patterns were then used to detect the photoadducts in DNA isolated from biologic samples (lymphocytes and keratinocytes) treated with clinically relevant doses of 8-MOP and UVA. In extracorporeal photopheresis, lymphocytes are exposed to 1–2 J/cm² UVA and 8-MOP concentrations ranging from 50–250 ng/ml [3]. In PUVA, the skin is exposed to UVA doses which range from 1 J/cm² to 15 J/cm². The 8-MOP concentration present in the epidermis of guinea pigs after ingestion of the drug has been estimated to be 68% of the level in plasma [10]. Studies performed in our laboratory have shown that the average 8-MOP level in human plasma is 125 ng/ml [2]. Therefore, our treatment of keratinocytes with 50 ng/ml of 8-MOP and 3 J/cm² falls within the range of in vivo PUVA conditions.

Our results show that treatment of lymphocytes and keratinocytes with 8-MOP and UVA results in the formation of three major photoproducts: the 4′,5′-monoadduct, the 3,4-monoadduct, and the crosslink. In lymphocytes treated with 100 ng/ml 8-MOP, 80% of the photoreaction occurs at the 4′,5′ site, resulting in formation of either the 4′,5′-monoadduct or the crosslink. This distribution is consistent with other studies [11]. In preliminary studies performed at a lower concentration of 8-MOP (10 ng/ml), photoreaction at the 4′,5′ site is not as favored and there is almost a twofold increase in the formation of the 3,4-monoadduct (data not shown). Apparently some of the high-affinity 8-MOP binding sites may be more suitable for 3,4-monoadduct formation.

In keratinocytes treated with 50 ng/ml of 8-MOP and 3 J/cm² UVA, crosslinks formed to a lesser extent and the 3,4-monoadduct, to a greater extent when compared to lymphocytes treated with 100 ng/ml of 8-MOP and 1 J/cm² UVA. This difference in the distribution of adducts in different cells, both of which are the targets of 8-MOP/UVA photochemotherapy, highlights the importance of studying the effects of each of these photoadducts on properties such as repair (see above), mutagenesis [13], and persistent photosensitivity [14] in biologically relevant cells. As seen from the above examples, a particular cell may have different photoadduct distributions.

Table I. Photoadduct Distribution in Poly (dA-dT)

Wavelength	Percent 3,4-MA	Percent 4′,5′-MA	Percent XL
400 nm	14.5	66.0	19.5
400 nm → 350 nm[a]	35.4	4.6	60.0

[a] 400 nm radiation was used initially; after precipitation and washing to remove unbound 8-MOP, the sample was exposed to 350 nm radiation. The bandwidth of the monochromator at each wavelength was 10 nm.

Table II. 8-MOP Photoadduct Distribution in Cells

8-MOP/UVA	Percent 3,4-MA	Percent 4′,5′-MA	Percent XL
Lymphocytes[a]	19.9 ± 6.2	42.2 ± 3.8	37.9 ± 2.4
Keratinocytes[b]	33.9 ± 3.9	36.5 ± 2.4	26.5 ± 5.0

[a] 100 ng/ml; 1 J/cm² (analysis of duplicate experiments).
[b] 50 ng/ml; 3 J/cm² (analysis of triplicate experiments).

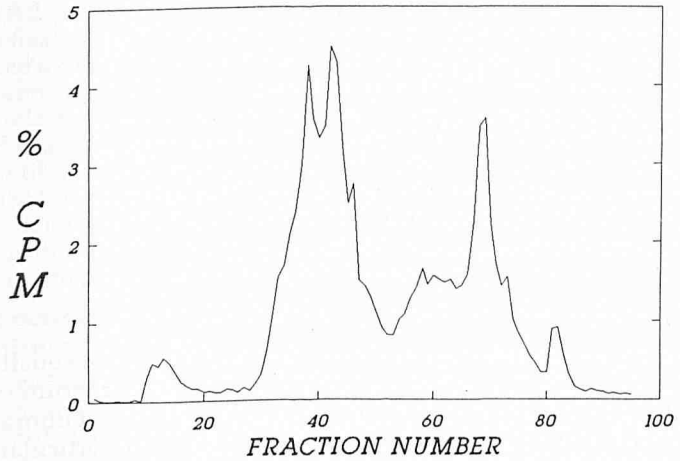

Figure 5. HPLC analysis of DNA from 8-MOP/UVA-treated keratinocytes. Percent of total CPM versus fraction number.

It is also shown in this study that 8-MOP crosslinks form in poly(dA-dT) exposed to 400 nm radiation. Several investigators, assuming that only monoadducts form at this wavelength, have designed experiments using 400 nm light to study the effects of monoadducts on cells [14]. In fact, as shown in the *Results,* irradiation of monoadducted poly(dA-dT) leads to the formation of 3,4-monoadducts as well as crosslinks (see Fig 3*B*). Therefore, depending on the type of cells and exact irradiating wavelengths used, it may not be correct to attribute the effects of secondary UVA exposures to crosslink formation alone. Our study suggests that before conclusions can be drawn regarding the effects of monoadducts alone (or monoadducts converted to crosslinks by additional UVA exposure), it is important to verify that crosslinks have not formed in a particular cell line under the irradiation conditions employed. Furthermore, the extent of 4′,5′-monoadduct conversion to crosslink should be quantified prior to attributing cellular effects to specific molecular entities.

Additional studies in our laboratory have shown that 25% of 8-MOP photoadducts are removed within 48 h [9]. These experiments showed that the ability of lymphocytes to remove 8-MOP photoadducts was dose dependent. An 8-MOP concentration of 100 ng/ml in combination with 1 J/cm^2 UVA blocked the removal of photoadducts. At lower concentrations adduct removal was accompanied by the recovery of proliferative activity upon exposure to phytohemagglutinin. In future studies the techniques described in this report will be used to characterize the repair of the three 8-MOP photoadducts.

Recent work from several laboratories has implicated cellular molecules other than nuclear DNA as targets for 8-MOP photomodification [15,16]. For example, in lymphocytes treated with 8-MOP and UVA, cell membrane DNA is modified at one tenth the efficiency of nuclear DNA [15]. Similar studies have not been performed in keratinocytes. Thus, new photoadducts with lipids and cell membrane DNA, acting in concert with the nuclear DNA photoadducts, may combine to produce the therapeutic responses seen in a wide variety of diseases amenable to 8-MOP and UVA photochemotherapy.

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